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Neuregulin1 administration increases axonal elongation in dissociated primary sensory neuron cultures

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Abstract

Neuregulin1 is a family of growth and differentiation factors involved in various functions of both peripheral and central nervous system including the regenerative processes that underlie regeneration of damaged peripheral nerves. In the present study we tested in vitro the effect of Neuregulin1 administration on dissociated rat dorsal root ganglion (DRG). Activity of neuregulin1 was compared to the activity of nerve growth factor in the same in vitro experimental model. Results showed that neurite outgrowth is enhanced by the addition of both neuregulin1 and nerve growth factor to the culture medium. While neuregulin1 was responsible for the growth of longer neurites, DRG neurons incubated with nerve growth factor showed shorter and more branched axons. Using enzyme-linked immunosorbent assay we also showed that the release of nerve growth factor, but not of brain derived neurotrophic factor is improved in DRG neuron treated with neuregulin1. On the other hand, the assay with growth factor blocking antibody, showed that effects exerted by neuregulin1 on neurite outgrowth is only partially due to the release of nerve growth factor. Taken together the results of this study provide a better understanding on the role of neuregulin1 in sensory neurons.

Introduction

Peripheral nerve damage due to trauma, tumour surgery or dis-ease represents a relevant social and economical problem [1–3]. Unlike the central nervous system, the peripheral nerve has a strong potential for regeneration [4]. If an appropriate microenvironment is provided, the damaged axons extend their processes achieving a variable degree of regeneration and ensuring a variable degree of recovery of motor and sensory functions. The ability of injured nerves to regenerate across the site of lesion and recover functional connections with their target organs depends on several variables, including the age of the patient and the type and the severity of the lesion [5]. Microsurgical reconstruction is generally carried out for nerve repair, especially in case of severe lesions such as nerve transections. However no re-pair technique ensures a complete recovery of motor and sensory

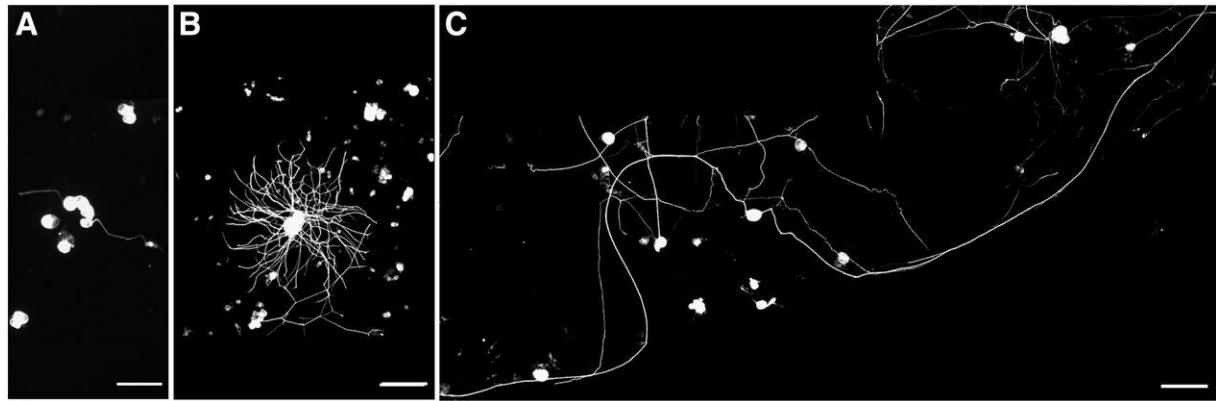


Fig. 1 – Immunocytochemical staining for β III tubulin (FITC) of DRG neurons following 72 h of culture with BSM (negative control, A), BSM with addition of 1 μ g/ml NGF (B) and 50 ng/ml NRG1 (C) respectively. Scale bar = 100 μ m.

functions, especially when nerve trauma is characterized by substantial loss of tissue [6]. Therefore, in order to enhance nerve re-covery, new therapeutic strategies are needed to enhance axonal regeneration based on a better understanding of the cellular and molecular biology of nerve regeneration.

Schwann cells (SCs) are the glial cell type of the peripheral nervous system; they enwrap all axons of peripheral nerves and play a critical role in the nerve regeneration process by providing trophic, tropic and structural support to regenerating axons. After a peripheral nerve injury, the axons in the distal nerve stump degenerate (Wallerian degeneration) while SCs dedifferentiate and actively proliferate. As the axon regenerate, new SCs promote neurite outgrowth by releasing various substances, such as cell adhesion molecules, integrins and neurotrophic factors [2,5,7,8]. These growth latter factors are a group of peptides that during development are involved in the promotion of neuronal survival and proliferation while, following nerve injury, they support axonal outgrowth [4,5,9].

A number of studies have shown that two neurotrophic factors, namely nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), are involved in peripheral nerve regenerating processes [4,5,9,10]. Both NGF and BDNF are expressed and released by SCs, they are produced by SCs during injury, form a diffused gradient around regenerating axons and appear to have complementary roles in peripheral nerve regeneration.

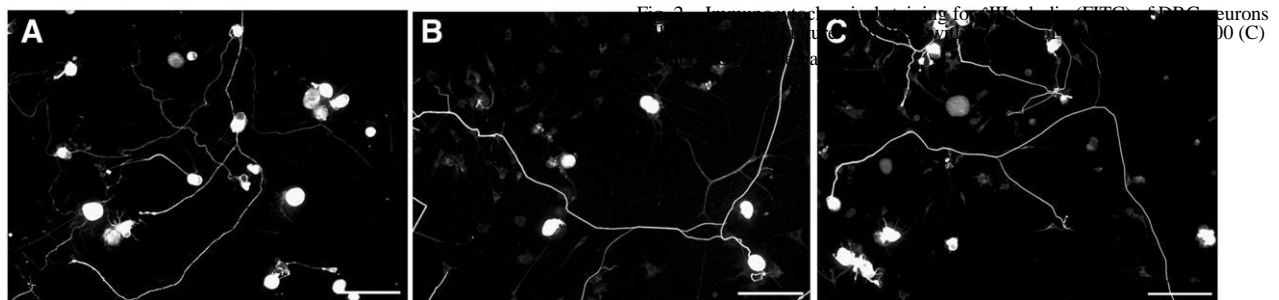
Previous studies have also demonstrated the important role of the gliotrophic system based on neuregulin1 (NRG1) signalling in peripheral nerve regeneration after different types of nerve injuries [11–14]. NRG1 is a family of proteins encoded by a single gene that, through the existence of several promoters and alternative splicing, is expressed into more than 20 different isoforms [15,16]. Some of these isoforms are known to be strongly involved in the regulation of myelination in the peripheral nervous system [17,18] and the pro-motion of gliogenic fate of neural crest cells during development [19,20]. Other isoforms promote the dedifferentiation and migration of SCs after nerve lesion and their subsequent proliferation and survival mediated by axonal signalling [21–23].

The biological effects of all NRG1 isoforms are exerted through their interaction with two members of the EGF-receptor family, namely ErbB3 and ErbB4. Also ErbB2 plays a role in NRG1/ErbB

transduction pathway since it functions as a co-receptor for ErbB3 and ErbB4 [24,25]. In peripheral nervous system, data on the expression of ErbB2, ErbB3 and NRG1 in both SCs and dorsal root ganglia (DRG) neurons have been reported [21,26,27]. In addition, the modulation of the NRG1/ErbB system has been demonstrated during the early stages of nerve regeneration after both severe nerve injuries characterized by substance loss [12,14,28] and injuries where nerves continuity is re-established by means of end-to-end or end-to-side neurorrhaphy [11].

These findings raise the possibility that the delivery of NRG1 in the site of injury could be useful in the promotion of peripheral nerve regeneration.

However, several crucial issues are still to be clarified prior to considering NRG1 as a potential target for designing new therapeutic regenerative strategies. Since the complexity of the *in vivo* conditions makes it difficult to study the cellular and molecular mechanisms that regulate axonal growth, a useful alternative approach is an *in vitro* model of dissociated DRG neurons. As adult DRG neurons survive in the absence of added trophic factors [10], they are a useful model to study the mechanisms of peripheral nerve regeneration and the effects of various experimental treatments on neuronal survival and axonal growth [4,29–31]. In this study we tested the effect of NRG1-treatment on dissociated DRG neurons in comparison to NGF treatment and basic culture control conditions on this *in vitro* model. Among the different isoforms, [15,16], the NRG1 β 1 was chosen due to its known effect on neurons [32].



Materials and methods

DRG neuron harvest

All animal procedures were carried out in compliance with UK Animals (Scientific Procedures) Act 1986. DRG neurons were harvested from adult male Sprague–Dawley rats using a previously described protocol [33]. Dissociated neurons were resuspended in modified Bottenstein and Sato's medium (BSM; F12 medium containing 100 μ M putrescine, 30 nM sodium selenite, 20 nM progesterone, 1 mg/ml BSA, 0.1 mg/ml transferrin, 0.01 mM cytosine arabinoside and 10 pM insulin (all Sigma)). The DRG neurons were seeded on glass coverslips in 24 well plates pre-coated with 2 μ g/ml laminin (Sigma) [34].

After 2 h BSM (negative control), BSM supplemented with either of NRG1 β 1 (with EGF domain) 20, 50, 100 ng/ml (R&D Systems, UK), NGF (1 μ g/ml, Sigma) or anti-NGF antibodies (1 μ g/ml, Millipore UK) were added to DRG culture which were incubated at 37 °C, 5% CO₂ for 24 h or 72 h for ELISA and immunocytochemistry.

Immunocytochemistry

After 72 h in culture, the dissociated DRG neurons were fixed in 4% (w/v) paraformaldehyde (20 min,

RT). Samples were immuno-stained for anti- β III tubulin (monoclonal, 1:1000, Sigma-Aldrich) and secondary antibodies FITC-conjugated horse anti-mouse (1:100, Vector Laboratories, UK). The samples were mounted with Vectashield™ (Vector Labs, UK) and examined under a fluorescence microscope (Olympus BX60) capturing, for each treatment, images from randomly selected ten sampling fields for quantification (10 \times and 20 \times magnifications). Image analysis was carried out using ImageJ Imaging software (<http://rsbweb.nih.gov/ij/index.html>). Additional immunocytochemical staining with no primary antibody was carried out as a negative control. The number of β III tubulin-positive and β III tubulin-negative cells was counted, in an area of 2.5 mm², in triplicate for three independent experiments.

Neurite outgrowth analysis

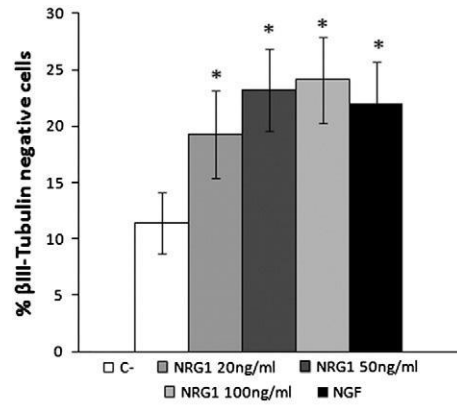
Three independent culture experiments were carried out for each experimental condition. In the sampling fields, neurite outgrowth was assessed in all cells that were anti- β III tubulin positive. The following four separate parameters were analysed: number of processes grown from each neuron, length of longest neurite, total neurite area and the percentage of process-bearing neurons (neurite sprouting) [33]. All measurements were carried out using ImageJ tools. The parameters were measured after a 72 h period as a later incubation times the neurite outgrowth from neighbouring neurons was too extensive and crossing over each other, making reliable measurement impossible.

Enzyme-linked immunosorbent assay (ELISA)

DRG neurons were cultured in the presence of BSM, 50 ng/ml NRG1 or 1 μ g/ml NGF. After 24 h and 72 h the medium was collected and analysed by ELISA using the ChemiKine™BDNF or NGF sandwich ELISA kits (Millipore Chemicon, UK) according to the manufacturer's protocol. All samples were analysed in triplicate and the absorbance was measured at 450 nm (Multiskan MC plate reader, Labsystems, UK).

Statistical analysis

Statistical analysis for DRG cultures was conducted using SPSS software (IBM SPSS Inc., USA). The data are expressed by mean \pm standard deviation. ANOVA and Bonferroni's Multiple Comparison Test tests were used to compare data from different groups. For the statistical analysis of the number of β III tubulin-positive and -negative cells Mann–Whitney test was used.



Fig

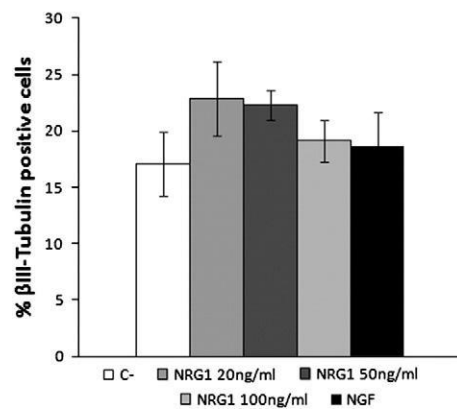


Fig. 3 – Quantification of βIII tubulin-positive and βIII tubulin-negative cells expressed in percentage of the total number of cells after 72 h of culture in the presence of BSM (negative control), BSM added with 20, 50, 100 ng/ml NRG1, and BSM added with 1 μg/ml NGF. Treatments vs. negative control: *P < 0.05.

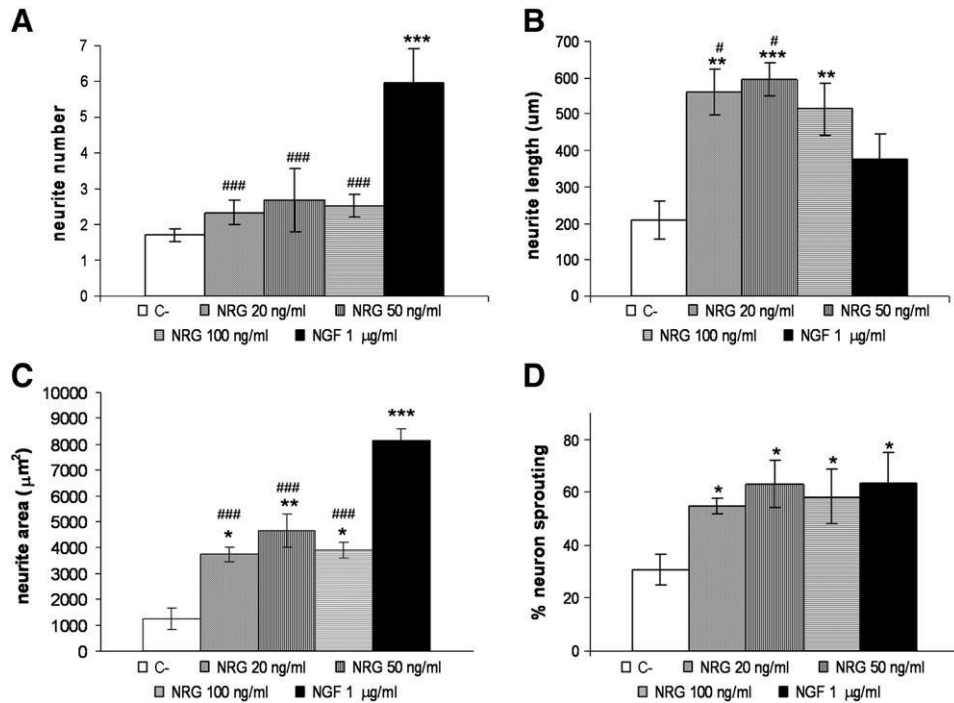


Fig. 4 – Quantification of DRG neurite outgrowth following 72 h of culture in the presence of BSM (negative control), BSM added with 20, 50, 100 ng/ml NRG1, and BSM added with 1 µg/ml NGF. Four parameters were measured: neurite number (A), length of longest neurite (B), total neurite area (C), and percentage of neurons sprouting neurites (D). Treatments vs. negative control:

*P < 0.05, **P < 0.01, ***P < 0.001; NRG1 vs NGF #P < 0.05 ###P < 0.001.

Results

NRG1 and NGF treatment influences differently neurite outgrowth from DRG neurons

Neurite outgrowth from DRG neurons were immunostained with β III tubulin antibodies and quantification was carried out following five different experimental conditions: BSM alone (Fig. 1A), BSM + 1 µg/ml NGF (Fig. 1B), and BSM + 20, 50, 100 ng/ml NRG1 (Figs. 1C, 2A–C). The number of β III tubulin-positive and β III tubulin-negative cells is reported in Fig. 3. Results showed that NRG1 treatment, as well as NGF treatment, increased the number of non-neuronal cells while it did not influence neuron number.

The addition of either NGF or NRG1 to the culture medium induced evident changes in neurite outgrowth of DRG neurons in comparison to BSM controls (Fig. 1). It was also clear that the effects of NRG1 and NGF were different. While NGF promoted the elongation of numerous and short neurites (Fig. 1B), consistently with the results of previous studies [4,35], NRG1 (Figs. 1C and 2A–C) induced the elongation of fewer but much longer neurites.

Quantitative analysis was consistent with the qualitative observations and showed that NGF treatment induced a significant increase ($P < 0.001$) of neurite number in comparison to control, while none of the NRG1 doses applied influenced significantly the number of neurites originating from each neuron, this being significantly low (Fig. 4A).

Quantitative analysis of the neurite length (Fig. 4B) showed that the addition of 20 or 50 ng/ml NRG1 increased significantly their values in comparison to the addition of either BSM controls (20 ng/ml NRG1 vs BSM $P < 0.01$; 50 ng/ml NRG1 vs BSM $P < 0.001$) or NGF ($P < 0.01$). The addition of the

highest dose of NRG1 (100 ng/ml), increased the neurite length to a statistically significant value ($P < 0.01$) was reached only in comparison to BSM controls.

The quantitative analysis of total neurite area (Fig. 4C) showed that the area values are significantly greater following the addition of NGF ($P < 0.001$) in comparison to the addition of NRG1 and BSM controls. Although significantly lower than NGF-treated DRG neurons ($P < 0.001$), all three NRG1-treated groups also showed a significant increased neurite area in comparison to BSM controls (20 and 100 ng/ml NRG1 vs BSM $P < 0.05$; 50 ng/ml NRG1 vs BSM $P < 0.01$).

There was no statistically significant difference between NRG1 and NGF treatment for the percentage of process-bearing neurons (Fig. 4D), while in comparison to BSM controls both NGF and NRG1 treatment increased the percentage of neurite sprouting ($P < 0.05$).

Release of NGF, but not of BDNF, increased following treatment with NRG1

We investigated whether the effects on neurite outgrowth observed in the presence of NRG1 may be mediated, at least in part, by an increase of NGF and BDNF release after NRG1 administration.

The release of NGF and BDNF in the medium was quantified after 24 h and 72 h of culture. Results showed an increased release of BDNF from cells cultured in the presence of NGF at both 24 ($P < 0.05$) and 72 h ($P < 0.001$) in comparison to untreated neurons (Figs. 5 and 6).

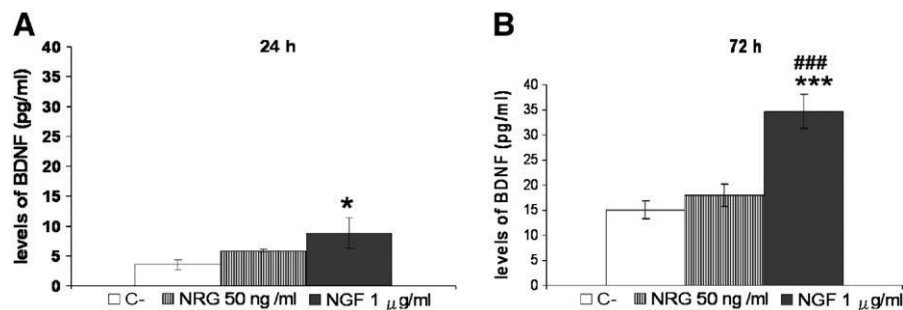


Fig. 5 – ELISA quantification of BDNF in DRG culture in the presence of BSM (negative control), BSM added with 50 ng/ml NRG1, and BSM added with 1 µg/ml NGF for 24 (A) and 72 (B) hours. NGF vs. C-: * $P < 0.05$, *** $P < 0.001$, NGF vs. NRG1 ^{###} $P < 0.01$.

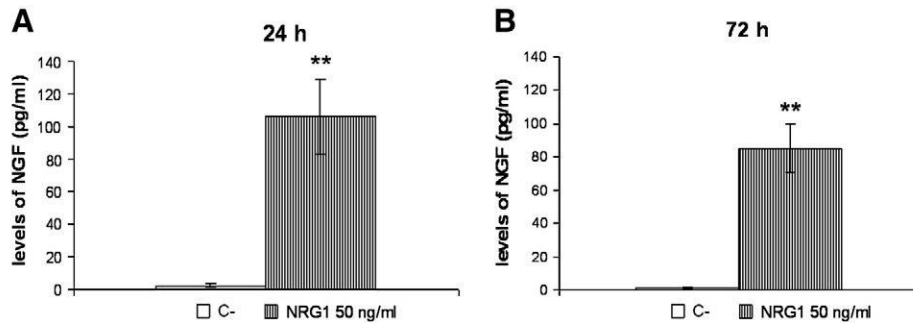


Fig. 6 – ELISA quantification of NGF in DRG culture in the presence of BSM (negative control) and BSM added with 50 ng/ml NRG1 for 24 (A) and 72 (B) hours. NRG1 vs. C-: **P < 0.01.

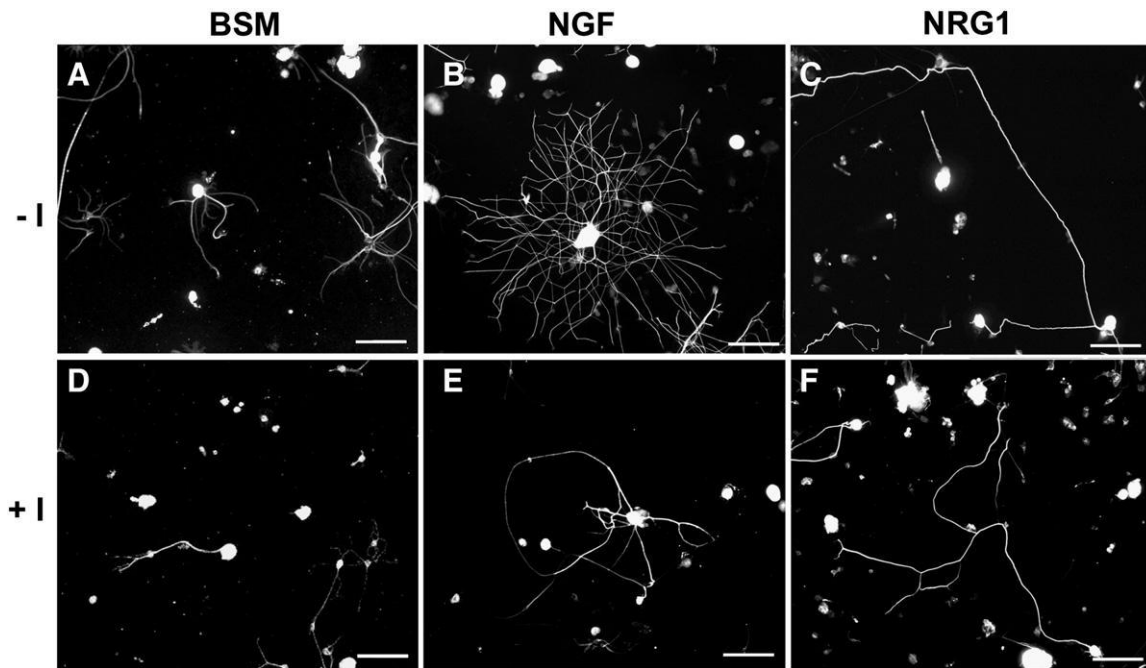


Fig. 7 – Immunocytochemical staining for β III tubulin (FITC) of DRG neurons following 72 h of culture with BSM (A, D), BSM added with 1 μ g/ml NGF (B, E) and BSM added with 50 ng/ml NRG1 (C, F) in the presence (+I) or absence (-I) of NGF blocking antibodies. Scale bar = 100 μ m.

NRG1 addition to culture medium induced no significant changes in BDNF release in comparison to controls (Fig. 5) while increased significantly the release of NGF at both 24 h and 72 h of culture ($P < 0.01$) (Fig. 6).

NRG1 effects on DRG neuron neurite outgrowth are partially reduced by NGF blocking antibodies

To verify if the effects of NRG1 on DRG cultures are mediated directly by its interaction with ErbB receptors, or are due to an indirect effect mediated by the release of NGF, experiments in the presence of NGF blocking antibodies were performed.

First, we administered NGF blocking antibodies to control DRG neuron cultures and we showed that it did not influence neuron survival (data not shown).

Then, we also verified that the addition of NGF blocking anti-bodies did not produce any significant changes in the four parameters evaluated (data not shown).

Finally, DRG neurons were cultured for 72 h in the presence of BSM alone or following the addition of either 50 ng/ml NRG1 or 1 μ g/ml NGF with or without NGF blocking antibodies. Results showed that addition of NGF blocking antibodies did not induce clear changes in control conditions (Figs. 7A, D) while it caused a blocking effect on the neurite outgrowth in all experimental conditions (Figs. 7B, C, E, F). Quantification of neurite number (Fig. 8A) showed that following NGF stimulation there was a significantly lower number of neurites in the presence of the NGF blocking antibodies ($P < 0.001$) even if the neurite number was still significantly higher ($P < 0.01$) than BSM controls.

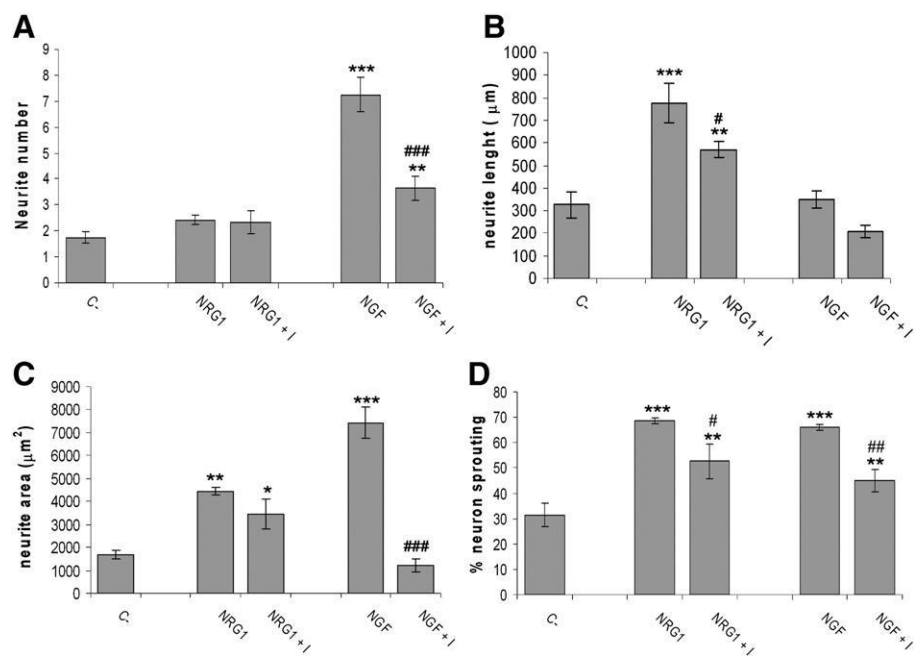


Fig. 8 – Quantification of DRG neurite outgrowth following 72 h of culture in the presence of BSM (C-), BSM added with 50 ng/ml NRG1 or 1 μ g/ml NGF in the presence or absence of NGF blocking antibodies (+ I). Four parameters were measured: neurite number (A), length of longest neurite (B), total neurite area (C) and percentage of neurons sprouting neurites (D). Treatments vs. negative control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; NRG1/NGF vs. NRG1/NGF added with NGF blocking antibody # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

NGF blocking antibodies induced a reduction in neurite length when DRG neurons were cultured with NRG1 ($P < 0.05$) but they did not produce any significant reduction when DRG neurons were cultured in the presence of NGF (Fig. 8B).

A significant ($P < 0.001$) reduction in total neurite area was detected when DRG neurons were cultured with NGF and NGF blocking antibodies (Fig. 8C). In particular, the addition of the inhibitor decreases the neuritic area to levels that were not statistically different from the control condition, in accordance with the results of previous studies [4]. By contrast, no significant changes in neurite area were observed in NRG1-treated DRG neurons after addition of NGF blocking antibody (Fig. 8C).

Finally, the results of sprouting neurons percentage (Fig. 8D) showed that the addition of NGF blocking antibodies induced a significant reduction in both NRG1 ($P < 0.05$) and NGF-treated ($P < 0.01$) neurons.

Discussion

It is acknowledged that NRG1 exert a broad range of functions in glial cell development as well as in the maintenance of normal function of both peripheral and central nervous system during adult life [18,36]. Moreover, due to the existence of a wide range of NRG1 alternatively spliced forms, this large family of molecules have also been implicated in a variety of human diseases including peripheral neuropathies, multiple sclerosis, schizophrenia and cancer [36,37].

While the biological functions mediated by NRG1 are not still fully understood, it has been demonstrated that many of the

isoforms identified so far mediate a wide range of cell–cell communications through a paracrine mechanism of action [15]. During Wallerian degeneration that occurs following peripheral nerve injuries [38], NRG1 isoforms are released from axons in a regulated manner and mediate the spatially and temporally adjusted bidirectional communication between axons and their surrounding SCs [39].

In this study we further investigated the role of NRG1 on dissociated primary sensory neurons with particular emphasis on the neurite growth changes.

To address this issue, we have analysed the effects of NRG1 on DRG neurons in comparison to those induced by the administration of NGF, the effects of which on the regeneration of adult DRG axons are well known [4,10], and basic culture controls.

Our results showed that NRG1 have a different effect on neurite outgrowth in comparison to NGF.

The results of our study are novel because they were obtained on DGR dissociated cultures and not organotypic DRG explants or co-cultures [32,40,42,43].

Following treatment with NGF a significant increase in total neurite density and in the number of neurites developed from each neuron was detected, while the effect of NRG1 was mainly represented by a large increase in neurite elongation. This observation is in agreement with a previous study of Mahanthappa et al. who showed an increased neurite elongation in an organotypic model of explanted DRGs following treatment with GGF [38].

We also tested if the effects of NRG1 on neurite outgrowth were exerted directly or were mediated by other trophic factors, like NGF and BDNF [4,5]. We showed that the secretion of NGF but not of BDNF is enhanced by NRG1 treatment suggesting that the effects of NRG1 are at least in part mediated by NGF release. A possible explanation of this observation can be found in the hypothesis advanced by Esper and co-workers [36,41] who suggested that SCs release NGF that exerts its biological action through the activation of TrkA receptors expressed on the axonal membrane. The activation of TrkA receptors determines the sub-sequent activation of protein kinase C (PKC) which in turn rapidly promotes the phosphorylation and release of NRG1 from the axons. Therefore, NRG1 may exert a paracrine effect by binding to ErbB receptors on SCs inducing an increase of NGF secretion in the medium establishing a positive feedback [36,41]. This possibility is supported by the evidence that, in our experiments, non-neuronal cells increased significantly after NRG1 treatment which is a potent mitogen for glial cells.

To further investigate to which extent the observed effects were due mainly to a direct action of NRG1 on neurites or were indirectly mediated by the release of NGF, we added NGF inhibitor to the cell cultures and we detected a partial reduction on neurite sprouting and neurite length promoted by NRG1, suggesting that the biological effects of NRG1 on neurite outgrowth are partially mediated by NGF release.

The observation that NRG1 induces the growth of fewer but longer neurites in comparison to NGF, deserves particular attention in the perspective of peripheral nerve repair. In fact, since prevention of muscle atrophy and degeneration is a key issue for successful functional recovery after nerve injury [44], it is very important that the regenerated axons reach their target as much rapidly as possible. In this view, the effects, both direct or glial cell-mediated, on neurite elongation exerted by NRG1 could represent a very positive tool on axonal regeneration after nerve injury in vivo.

Acknowledgments

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